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U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
0136/0J067

BOX SE &

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

09/807234

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/KR00/01213	26 October 2000	15 March 2000

TITLE OF INVENTION

GENOTYPING KIT FOR DIAGNOSIS OF HUMAN PAPILLOMAVIRUS INFECTION

APPLICANT(S) FOR DO/EO/US

PARK, Tae-Shin; KIM, Sung-Keun; KIM, Jin-Hee; PARK, Mi-Sun

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.

3. [] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. [] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [X] A translation of the International Application into English (35 U.S. C. 371 (c)2)).

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).

9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 (with 14 references).

12. [X] An assignment document for recording. A **separate** cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [] A FIRST preliminary amendment.
    [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] A substitute specification.

15. [] A change of power of attorney an/or address letter.

16. [X] Other items or information: Affirmation of Priority Claim; Verified Statement Claiming Small Entity Status; Sequence Listing with Sequence Listing Diskette and Statement Pursuant To Rule 1.821(f).

EXPRESS MAIL CERTIFICATE

Date 4-6-01 Label No. EL853598812US

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

G KARASZI
Name (Print)

G Karaszi
Signature

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JC02 Rec'd PCT/PTO 06 APR 2001

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/807234	INTERNATIONAL APPLICATION NO.: PCT/KR00/01213	Attorney's Docket Number 0136/0J067

17. [x] The following fees are submitted:		ALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492 (a)(1)-(5)): Search Report has been prepared by the EPO [] or JPO []	$860.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$690.00		
No international preliminary examination fee paid to USPTO(37 CFR 4.482) but international search fee paid to USPTO (37 CFR 1.445 (a) (2)...	$710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	$1,000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....	$100.00	$1,000.00	

Surcharge of $130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).			ENTER APPROPRIATE BASIC FEE AMOUNT =	$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	11-20	0	X $18.00	$	
Independent Claims	3-3	0	X $80.00	$	
Multiple dependent claims(s) (if applicable)		+270		$	
			TOTAL OF ABOVE CALCULATIONS =	$1000.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$500.00	
			SUBTOTAL =	$500.00	
Processing fee of $130.00 for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)).			+		
			TOTAL NATIONAL FEE =	$500.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property			+	$40.00	
			TOTAL FEES ENCLOSED =	$540.00	
				Amount to be refunded	$
				charged:	$

a. [X] A check in the amount of $540.00 to cover the above fees is enclosed.

b. [] Please charge my Deposit Account No.04-0100 in the amount of $ to cover the above fees.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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NAME Marie L. Collazo, Registered Patent Agent
REGISTRATION NO. 44,085

0136/01067

Inventor or Patentee: PARK, Tae-Shin et al. Case No.: Page 1
Applicant or Patent No.:
Filed or Issued:
Title: **GENOTYPING KIT FOR DIAGNOSIS OF HUMAN PAPILLOMAVIRUS INFECTION**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS

1. I, the undersigned, do hereby declare that:

a. [ ] **I am an independent inventor** as defined in 37 CFR 1.9(c) for purposes of paying reduced fees to the Patent and Trademark Office with regard to the invention described in the patent or application identified above; OR

b. [ ] **While I am not an inventor, I declare that rights under contract or law have been conveyed to and remain with me with regard to the invention described in the patent or application identified above.** I would qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying fees to the United States Patent and Trademark Office if I had made the invention; OR

c. [ ] **I am the owner of the small business concern** identified below
OR
[X] **I am an official of the small business concern** empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS:
ADDRESS OF SMALL BUSINESS:

If either of the boxes in item (c) is checked, I further declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both. I further declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in the patent or application identified above; or

d. [ ] **I am an official empowered to act on behalf of the nonprofit organization** identified below:

NAME OF NONPROFIT ORGANIZATION: BIOMEDLAB CORPORATION
ADDRESS OF NONPROFIT ORGANIZATION: 4th Fl., Dongsung Bldg., 1-49 Dongsung-dong, Jongno-gu, Seoul 110-510, Republic of Korea

TYPE OF NONPROFIT ORGANIZATION:

[ ] university or other institution of higher education
[ ] tax exempt under Internal Revenue Service Code(26 USC 501(a) and 501(c)(3))
[ ] nonprofit scientific or educational under statute of state of the United States of America
(name of state:)
(citation of statute:)
[ ] would qualify as tax exempt under Internal Revenue Service Code(26 USC 501(a) and 501(c)(3)) if located in the United States of America
[ ] would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America
(name of state:)
(citation of statute:)

If Box(d) is checked, I further declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in the patent or application identified above.

2. The individual, concern or organization identified above has not assigned, granted, conveyed or licensed, and is under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

3. If the rights held by above-identified individual, concern or organization are not exclusive, each individual, concern or organization having rights in the invention are identified below. Each such individual, concern or organization must file separate verified statements averring to their status as small entities.

***Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).**

FULL NAME:
ADDRESS:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

FULL NAME:
ADDRESS:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

4. I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small-entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: KIM, Jong-Won
TITLE OF PERSON (if not an owner or individual): Representative
ADDRESS OF PERSON SIGNING: 4th Fl., Dongsung Bldg., 1-49 Dongsung-dong, Jongno-gu, Seoul 110-510, Republic of Korea

NAME OF PERSON SIGNING:
TITLE OF PERSON (if not an owner or individual):
ADDRESS OF PERSON SIGNING:

NAME OF PERSON SIGNING:
TITLE OF PERSON (if not an owner or individual):
ADDRESS OF PERSON SIGNING:

SIGNATURE: 김종원 DATE: March. 16, 2001

SIGNATURE: DATE:

SIGNATURE: DATE:

GENOTYPING KIT FOR DIAGNOSIS OF HUMAN PAPILLOMAVIRUS INFECTION

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a genotyping kit for diagnosis of human papillomavirus(HPV) infection, more specifically, to a genotyping kit for detecting human papillomaviruses from clinical samples of infected patients using a DNA chip, and a process for preparing the said DNA chip and a method for diagnosis of HPV infection using the said genotyping kit.

Backgound of the Invention

Uterine cancer includes cervical cancer, endometrial cancer, uterine sarcoma and the like. For cervical cancer, approximately 450,000 new cases occur worldwide each year and approximately 6,000 in Korea. Since the occurrence of cervical cancer(including cervical intraepithelial neoplasia) occupies 22.1% of total cancer cases in Korean women, the highest incidence with the second highest death rate, the prevention, diagnosis and treatment of cervical cancer are regarded as the most important issue in women's health.

Cervical cancer progresses through a precancerous stage, cervical intraepithelial neoplasia(CIN) known to be mainly caused by human papillomavirus(HPV) infection. Especially, infection by particular types of HPV raises the possibility of developing invasive disease. Over 70 genotypes of HPV have been identified since the recognition of HPV as the main etiological factor for cervical cancer. Certain HPV genotypes were selectively found in the lesions of specific location or progression stage, which rendered

the biological diversity of HPV infection realized. Among the HPV genotypes detected in the anogenital area, over 10 genotypes have been classified as the high-risk group that are associated with an elevated risk for developing cervical cancer. Based on these findings, characterization of the biological differences of HPV infection is considered to be of significant importance to the diagnosis and prevention of cervical cancer.

For the diagnosis of cervical cancer at its early stage, Pap smear test has been most commonly used which is a cytological test performed as follows: old cells removed from the outermost layer of cells from the surface of the cervix are stained and examined for histopathological characteristics of HPV infection including koilocytosis, formation of perinuclear halo in the epithelial cells. However, due to the low diagnostic efficiency(1 - 15%) of Pap test together with other limitations, additional methods such as colposcopy are necessary for more dependable diagnosis. Colposcopic screening can detect HPV infection up to 70% but has disadvantages including high cost of the equipment, the need for skilled interpreters, and incapability of determining HPV genotypes to distinguish between the high-risk and low-risk infection. Therefore, efforts have been made continuously to develop techniques for the detection of HPV and identification of HPV genotypes to supplement conventional screening methods for cervical cancer and its precursors including Pap test.

The methods for detection of HPV and identification of HPV genotypes can be classified into two groups, i.e., direct detection of HPV DNA and detection of amplified HPV DNA. The methods for direct detection of HPV DNA include liquid hybridization(Hybrid Capture kit by Digene Diagnostics, Silver Spring, MD, USA, www.digene.com), Sourthern blot and dot blot with HPV type-specific probes, filter in situ hybridization(FISH) and the like, and the methods for the detection of amplified DNA include type-specific PCR (polymerase chain reaction) and general-primer

PCR. In particular, genotype analyses of amplified HPV DNA by general primer sets are commonly performed by employing dot blot hybridization, microtiter plate hybridization, or line probe assay. Among these methods, liquid hybridization by Hybrid Capture and line probe assay following general-primer PCR have been considered most suitable for diagnostic purposes. The line probe assay can detect about 20 different HPV genotypes by immobilized oligonucleotide probes on a nitrocellulose membrane, however, it lacks reliability due to low sensitivity and difficulties in data interpretation. Commercialized Hybrid Capture kit can detect HPV DNA in clinical samples without PCR amplification and distinguish between high-risk and low-risk HPV groups. However, the fact that Hybrid Capture kit cannot identify the genotypes of infecting HPV limits accurate risk determination since the risk factor amongst the high-risk HPV is not the same, in other words, intermediate-risk types are included in the high-risk group. Moreover, the use of RNA probe may pose low stability of the kit, and also possibility of contamination cannot be excluded.

Under these circumstances, there have been strong reasons for exploring and developing a simple and accurate method for detection of HPV infection and identification of the genotype of infecting HPV.

Summary of the Invention

The present inventors have tried to detect HPV infection and identify the types of HPV by way of genotyping DNA from clinical samples and prepared an HPV genotyping kit comprising a DNA chip with probes that have nucleotide sequences complementary to the DNA of HPV, primers for amplifying DNA obtained from clinical samples by PCR, and means for labeling amplified DNA hybridized to the probes of the said DNA chip, and successfully detected HPV infection and identified genotypes of infecting HPV by

the aid of the genotyping kit in a simple and accurate manner.

A primary object of the present invention is, therefore, to provide a genotyping kit for diagnosis of HPV infection.

The other object of the invention is to provide a process for preparing the DNA chip contained in the HPV genotyping kit.

Another object of the invention is to provide a method for diagnosis of HPV infection using the HPV genotyping kit.

Brief Description of the Drawings

The above and the other objects and features of the present invention will become apparent from the following description given in the conjunction with the accompanying drawings, in which:

Figure 1 is a schematic representation of the types and positions of the probes on the DNA chip.

Figure 2a is a photograph showing the result of HPV 16 DNA analysis.

Figure 2b is a photograph showing the result of HPV 18 DNA analysis.

Figure 2c is a photograph showing the result of HPV 31 DNA analysis.

Figure 2d is a photograph showing the result of HPV 33 DNA analysis.

Figure 2e is a photograph showing the result of HPV 35 DNA analysis.

Figure 2f is a photograph showing the result of HPV 39 DNA analysis.

Figure 2g is a photograph showing the result of HPV 45 DNA analysis.

Figure 2h is a photograph showing the result of HPV

51 DNA analysis.

Figure 2i is a photograph showing the result of HPV 52 DNA analysis.

Figure 2j is a photograph showing the result of HPV 56 DNA analysis.

Figure 2k is a photograph showing the result of HPV 58 DNA analysis.

Figure 2l is a photograph showing the result of HPV 59 DNA analysis.

Figure 2m is a photograph showing the result of HPV 66 DNA analysis.

Figure 3a is a photograph showing the result of HPV 6 DNA analysis.

Figure 3b is a photograph showing the result of HPV 11 DNA analysis.

Figure 3c is a photograph showing the result of HPV 34 DNA analysis.

Figure 3d is a photograph showing the result of HPV 40 DNA analysis.

Figure 3e is a photograph showing the result of HPV 42 DNA analysis.

Figure 3f is a photograph showing the result of HPV 44 DNA analysis.

Figure 4a is a photograph showing the result of DNA analysis of sample number 43 using the DNA chip of the invention.

Figure 4b is a photograph showing the result of DNA analysis of sample number 46 using the DNA chip of the invention.

Figure 4c is a photograph showing the result of DNA analysis of sample number 47 using the DNA chip of the invention.

Figure 4d is a photograph showing the result of DNA analysis of sample number 51 using the DNA chip of the invention.

Figure 4e is a photograph showing the result of DNA analysis of sample number 52 using the DNA chip of the

invention.

Figure 4f is a photograph showing the result of DNA analysis of sample number 53 using the DNA chip of the invention.

Figure 4g is a photograph showing the result of DNA analysis of sample number 54 using the DNA chip of the invention.

Figure 4h is a photograph showing the result of DNA analysis of sample number 57 using the DNA chip of the invention.

Figure 4i is a photograph showing the result of DNA analysis of sample number 95 using the DNA chip of the invention.

Figure 4j is a photograph showing the result of DNA analysis of sample number 107 using the DNA chip of the invention.

Figure 4k is a photograph showing the result of DNA analysis of sample number 115 using the DNA chip of the invention.

Figure 4l is a photograph showing the result of DNA analysis of sample number 124 using the DNA chip of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The genotyping kit of the invention for diagnosis of human papillomavirus(HPV) infection comprises: a DNA chip with probes that have nucleotide sequences complementary to DNA of HPV; primers for amplifying DNA obtained from clinical samples by PCR; and, means for labeling amplified DNA hybridized with the probes of the said DNA chip. The DNA chip may further comprise position markers to locate probes, and staining or labeling is performed by using means for labeling comprising preferably biotin-binding material, most preferably, streptavidin-R-phycoerythrin which is a conjugate of a fluorophore and a protein with biotin-binding sites.

The process for preparing DNA chip contained in the said HPV genotyping kit comprises the steps of: preparing 5' terminal amine-linked DNA probes which have nucleotide sequences complementary to DNA of HPV; affixing the DNA probes thus prepared to an aldehyde-derivatized solid surface; and, reducing excessive aldehydes not reacted with amine.

The process for preparing DNA chip of the invention is described in more detail by the following steps.

Step 1: Preparation of probes

5' terminal amine-linked DNA probes that have nucleotide sequences complementary to the DNA of HPV are prepared: The nucleotide sequences of the probes are designed and synthesized to have nucleotide sequences complementary to the DNA of HPV, preferably the L1 region of HPV DNA, and the probes are prepared by linking amine group at 5' terminal of the nucleotide sequences which enables the probes to bind to aldehyde-derivatized solid surface.

Step 2: Affixture of probes

DNA probes prepared in Step 1 are affixed to an aldehyde-derivatized surface of a solid support, preferably glass. The probes are affixed to the surface of solid support via Schiff's base reaction between an aldehyde group on the surface of solid support and an amine group at 5' terminal of the probe under an environment of 30 to 40℃ and 70 to 100% humidity, while controlling the concentration of probes in a range of preferably 100 to 300 pmol/㎕, more preferably 200 pmol/㎕.

Step 3: Preparation of DNA chip

Excessive aldehydes not reacted with amine on the solid surface are reduced by employing a reducing agent of NaBH (sodium borohydride), finally to prepare DNA chip.

The method for diagnosis of HPV infection using HPV genotyping kit of the invention comprises the steps of: amplifying DNA obtained from clinical samples by PCR with primers of the HPV genotyping kit; applying the amplified DNA to the DNA chip to hybridize the amplified DNA with DNA probes of the DNA chip; and, detecting DNA bound on the surface of the DNA chip after labeling hybridized DNA.

The method for diagnosis of HPV infection using HPV genotyping kit of the invention is further illustrated by the following steps.

Step 1: Amplification of sample DNA

DNA obtained from clinical samples is amplified using the primers of HPV genotyping kit, where polymerase chain reaction(PCR) employing biotin-16-dUTP is carried out to give biotin-containing amplified DNA.

Step 2: Hybridization

Amplified DNA thus obtained is applied to the DNA chip of HPV genotyping kit and hybridized with the probes of the DNA chip.

Step 3: Detection

The amplified sample DNA hybridized with the probes are labeled with means for labeling and detected with a confocal laser scanner: Streptavidin-R-phycoerythrin is preferably used as means for labeling which is a conjugate of a fluorophore with a high extinction coefficient and a

protein with 4 biotin-binding sites, which enables high sensitivity detection of hybridized spots on the DNA chip by the confocal laser scanner.

HPV genotyping kit of the invention is an implement that can detect HPV infection in a simple and accurate manner, as well as identify the types of infecting HPV, therefore, it may contribute to early diagnosis, prevention and treatment of cervical cancer.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention. Particularly, though DNA chip with 19 probes was prepared in the Examples described below, it is to be understood that the present invention is not limited by types and numbers of probes, but DNA chips using nucleotide sequences derived from HPV DNA and any variety of detection kits using the said DNA chips are intended to be included within the scope of the invention.

Example 1: Preparation of DNA chip

Prevalent HPV types including 13 high-risk types(HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66) and 6 low-risk types(HPV type 6, 11, 34, 40, 42, 44) were selected, and genotype-specific probe for each HPV type possessing amine group at 5' terminal of the sequence was prepared for the detection of HPV genotypes. The nucleotide sequence of each probe is as follows:

HPV 16: 5'-gtcattatgtgctgccatatctacttcaga-3'(SEQ ID NO: 1),
HPV 18: 5'-tgcttctacacagtctcctgtacctgggca-3'(SEQ ID NO: 2),
HPV 31: 5'-tgtttgtgctgcaattgcaaacagtgatac-3'(SEQ ID NO: 3),
HPV 33: 5'-tttatgcacacaagtaactagtgacagtac-3'(SEQ ID NO: 4),
HPV 35: 5'-gtctgtgtgttctgctgtgtcttctagtga-3'(SEQ ID NO: 5),
HPV 39: 5'-tctacctctatagagtcttccataccttct-3'(SEQ ID NO: 6),
HPV 45: 5'-acacaaaatcctgtgccaagtacatatgac-3'(SEQ ID NO: 7),
HPV 51: 5'-agcactgccactgctgcggtttccccaaca-3'(SEQ ID NO: 8),

HPV 52: 5'-tgctgaggttaaaaaggaaagcacatataa-3'(SEQ ID NO: 9),
HPV 56: 5'-gtactgctacagaacagttaagtaaatatg-3'(SEQ ID NO: 10),
HPV 58: 5'-attatgcactgaagtaactaaggaaggtac-3'(SEQ ID NO: 11),
HPV 59: 5'-ctgtgtgtgcttctactactgcttctattc-3'(SEQ ID NO: 12),
HPV 66: 5'-ctattaatgcagctaaaagcacattaacta-3'(SEQ ID NO: 13),
HPV 6: 5'-atccgtaactacatcttccacatacaccaa-3'(SEQ ID NO: 14),
HPV 11: 5'-atctgtgtctaaatctgctacatacactaa-3'(SEQ ID NO: 15),
HPV 34: 5'-tacacaatccacaagtacaaatgcaccata-3'(SEQ ID NO: 16),
HPV 40: 5'-gctgccacacagtcccccacaccaacccca-3'(SEQ ID NO: 17),
HPV 42: 5'-ctgcaacatctggtgatacatatacagctg-3'(SEQ ID NO: 18),
HPV 44: 5'-gccactacacagtcccctccgtctacatat-3'(SEQ ID NO: 19),

DNA chip was prepared as follows: each probe prepared above was dissolved in 3X SSC(45mM sodium citrate, 0.45M NaCl, pH 7.0) at a concentration of 200 pmol/㎕, and spotted onto an aldehyde-derivatized silylated slide(CSS-100, CEL, Houston, TX, USA) to form an array of spots with size of 150㎛ at 300㎛ spacing between spots using a microarrayer(GMS 417 Arrayer, TakaRa, Japan), followed by performing Schiff's base reaction under an environment of 37℃ and over 70% humidity for 4 hours. The slide was washed with 0.2%(w/v) sodium dodecyl sulfate(SDS), and with triple distilled water. Then, the slide was treated with NaBH solution(0.1g $NaBH_4$, 30ml phosphate buffered saline (PBS), 10ml ethanol) for 5 minutes to reduce excessive aldehydes not reacted with amine, followed by washing with triple distilled water and air-drying.

Example 2: Preparation of samples

In order to detect HPV infection in human cervical swabs, DNA was extracted from the said specimen and then purified. To test the adequacy of sample DNA, the said purified DNA was PCR amplified with beta-globin primers, PC03(5'-acacaactgtgttcactagc-3', SEQ ID NO: 20) and 5'-biotin linked-PC04(5'-caacttcatccacgttcacc-3', SEQ ID NO: 21). The DNA samples which reveal beta-globin DNA

amplification were selected and used for further analyses of HPV DNA.

As HPV DNA standards, plasmid DNA comprising HPV sequence obtained from the following distributors were used: HPV types 6, 11, 40, 45, and 51 from Dr. Ethel-Michele de Villiers, Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69009 Heidelerg, Germany; HPV types 35, 44, and 56 from Dr. Attila Lorincz, Vice President, R&D and Scientific Director, Digene Diagnostics, Inc., 2301-B Broadbirch Drive, Silver Spring, MD 20904, USA; HPV types 42, 58, and 59 from Dr. Toshihiko Matsukura, Department of Pathology and Laboratory of Pathology, AIDS Research Center, National Institute of Infectious Disease, Tokyo 162, Japan; HPV types 33, 34, 39, 52, and 66 from Dr. Gérard Orth, Unité Mixte Institut Pasteur/INSERM(U. 190), Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France.

Additionally, DNA extracted and purified from following cell lines were used as positive controls: SiHa cell line(HPV 16, KCLB 30035, Human squamous carcinoma, cervix) and HeLa cell line(HPV 18, KCLB 10002, Human epithelial carcinoma, cervix) which were purchased from Korean Cell Line Bank(Seoul National University, College of Medicine, Seoul, Korea).

Selected sample DNA described above were PCR amplified using the following primer sets: GP5$^+$(5'-tttgttactgtggtagatactac-3', SEQ ID NO: 22) and biotin-linked GP6 , Bio-GP6$^+$(5'-Biotin-gaaaaataaactgtaaatcatattc-3', SEQ ID NO: 23), and, GP5d$^+$(5'-tttkttachgtkgtdgatacyac-3', SEQ ID NO: 24) and GP6d$^+$(5'-gaaahataaaytgyaadtcataytc-3', SEQ ID NO: 25). The modified primer set GP5d$^+$/GP6d$^-$ was developed to facilitate PCR amplification of HPV DNA from clinical samples. In describing the nucleotide sequence, 'k' is employed to mean 'g' or 't', 'h' is 't', 'a', or 'c', 'd' is 'a', 't', or 'g', and 'y' is 't' or 'c'.

Example 2-1: Preparation of positive control samples

To obtain biotin-labeled amplified DNA samples, HPV 16 and HPV 18 DNA purified above were amplified by PCR with primers, GP5- and Bio-GP6-. PCR was performed in a 50 ㎕ of reaction mixture containing PCR buffer(50 mM KCl, 4 mM MgCl , 10 mM Tris-HCl, pH 8.3), 0.1 ㎍ of DNA, 4.5 mM $MgCl_2$, 50 pmol of each primer, 40 μ M each of dATP, dCTP, dGTP(Pharmacia), 30 μ M of dTTP(Pharmacia), 10 μ M biotin-16-dUTP(Boenringer Manheim, Germany) and 1 unit of *Taq* polymerase(TaKaRa, Japan) with 40 cycles of denaturation for 1 min at 94℃, primer annealing for 2 min at 40℃, and extension for 1 min 30sec at 72℃.

Example 2-2: Preparation of HPV standards

Biotin-linked amplified HPV DNA samples were prepared anologously as in Example 2-1, except for employing templates of various HPV plasmids described above.

Example 2-3: Preparation of sample DNA from clinical samples

Biotin-linked amplified DNA samples were obtained analogously as in Example 2-1, except that DNA obtained from uterine cervical swabs were used as templates, GP5d and GP6d were employed as primers, and PCR was performed with 40 cycles of denaturation for 1 min at 94℃, primer annealing for 2 min at 55℃, and extension for 1 min 30 sec at 72℃.

Example 3: Detection of HPV infection using DNA chip

Amplified DNA samples obtained in Example 2 were applied to the DNA chip prepared in Example 1, and hybridization was carried out in a hybridization reaction chamber made up of the Cover slip(GRACE Bio-Labs, USA,

PC4L-1.0) with 100 ㎕ capacity.

As for the quantity of hybridization reaction samples, 10 ㎕ each of amplified product was used for positive controls and plasmid DNA, and a mixture of 10 ㎕ of HPV amplified product and 5 ㎕ of beta-globin amplified product was used for DNA obtained from cervical swabs. The said reaction samples were denatured by adding 3N NaOH solution(10% v/v) and standing for 5 min at room temperature, and neutralized by adding 1 M Tris-HCl(pH 7.2, 5% v/v) followed by 3N HCl(10% v/v) and cooling for 5 min on ice. The samples were then mixed with a hybridization solution made up of 6X SSPE(saline-sodium phosphate-EDTA buffer, Sigma Chemical Co., St. Louis, MO, USA) and 0.2% SDS(sodium dodecyl sulfate), and applied onto the DNA chip. Hybridization reaction was carried out for 2 hours at 40℃, followed by washing with 3X SSPE for 2 min, 1X SSPE for 2 min, and air-drying at room temperature. The DNA chip hybridized with sample DNA was stained with a mixture of 5 ㎕ of streptavidin-R-phycoerythrin conjugate(50 ㎍/ml) and 95 ㎕ of 3X SSPE for 25 min, washed with 1X SSPE, and then analyzed for fluorescent signals(extinction 480 nm, emission >520 nm) by using a confocal laser scanner(GMS 418 Array Scanner, TaKaRa, Japan)(see: Figure 1, Figures 2a-2m, 3a-3f, and 4a-4l). Figure 1 is a schematic representation of the type and position of the probes on DNA chip: each number indicates each HPV probe, 'bg' indicates beta-globin probe placed to verify proper performance of hybridization reaction, 'M' indicates position marker for locating probes, open circles(o) indicate HPV and beta-globin probe-affixed positions, and closed circles(●) indicate positions of 'M'. Figures 2a-2m are photographs showing the results of high-risk group HPV DNA analyses using HPV plasmids and cervical cancer cell lines: Figure 2a is a photograph showing the result of HPV 16 DNA analysis, Figure 2b, the result of HPV 18 DNA analysis, Figure 2c, the result of HPV 31 DNA analysis, Figure 2d, the result of HPV 33 DNA analysis, Figure 2e, the result of HPV 35 DNA analysis, Figure 2f,

the result of HPV 39 DNA analysis, Figure 2g, the result of HPV 45 DNA analysis, Figure 2h, the result of HPV 51 DNA analysis, Figure 2I, the result of HPV 52 DNA analysis, Figure 2j, the result of HPV 56 DNA analysis, Figure 2k, the result of HPV 58 DNA analysis, Figure 2l, the result of HPV 59 DNA analysis, and Figure 2m, the result of HPV 66 DNA analysis. Figures 3a-3f are photographs showing the results of low-risk group HPV DNA analyses using HPV plasmids: Figure 3a is a photograph showing the results of HPV 6 DNA analysis, Figure 3b is a photograph showing the result of HPV 11 DNA analysis, Figure 3c, the result of HPV 34 DNA analysis, Figure 3d, the result of HPV 40 DNA analysis, Figure 3e, the result of HPV 42 DNA analysis, and Figure 3f, the result of HPV 44 DNA analysis. As shown in Figures 2a-2m and Figures 3a-3f, hybridization signals produced by the amplified DNA of HPV plasmid standards and HPV positive controls(cervical cancer cell lines) were observed clearly on the corresponding probes without significant cross-hybridization.

Example 4: Detection of HPV infection in clinical samples using DNA chip

In order to examine the accuracy and efficiency of diagnosis by the DNA chip of the invention, clinical samples were PCR amplified with primers comprising nucleotide sequences set forth in SEQ ID NO: 20-25, and then, for proper samples, diagnostic procedure using the DNA chip was performed to detect HPV infection as well as to determine the type of the infection.

DNA isolated from 124 specimens from uterine cervix were amplified using the method described in Example 2-3, and analyzed for HPV infection by using the DNA chip of the invention as described in Example 3. The above 124 isolated DNA were subjected to PCR-RFLP(Restriction Fragment Length Polymorphism) assay in which DNA was amplified, treated with restriction enzyme Ava II, Afa I,

Bgl II, Acc I or Ava I, and then the pattern of fragmentation produced by the restriction enzymes was analyzed to determine 6 types of HPV infection(HPV 16, 18, 31, 33, 52 and 58). The results of PCR-RFLP were confirmed by employing type-specific PCR technique(see: Hwang, T., J. Kor. Med. Sci., 15:593-599, 1999; Fujinaga, Y. et al., J. General Virology, 72:1039-1044, 1991). The results of the two methods, DNA chip of the invention and PCR-RFLP followed by type-specific PCR, were compared to determine diagnostic efficiency of the DNA chip method(see: Figures 4a-4l, Table 1). Figures 4a-4l are photographs showing Examples of the results of DNA chip analyses of cervical swab specimens for HPV infection. As shown in Figures 4a-4l, detailed diagnoses of HPV infection in the clinical samples via accurate detection and genotyping of the infecting HPV were successfully accomplished by using the DNA chip of the invention. The degree of agreement of the two methods was also measured for the above 124 cases of clinical specimens(see: Table 1).

Table 1. Comparison of the detection/genotyping results obtained by the invented genotyping kit and PCR-RFLP assay of prior art

Sample Nos.	Detection/Genotyping by Genotyping Kit	Detection/Genotyping by PCR-RFLP Assay
34	-	-
35	-	-
36	-	HPV 16
37	HPV 16, 56	HPV 16
38	HPV 58	HPV 58
39	HPV 56, 58	HPV 33
40	HPV 16	HPV 16
41	HPV 16	HPV 18
42	HPV 16	HPV 16
43	HPV 16	HPV 16
46	HPV 16	HPV 16
47	HPV 16	HPV 16
48	HPV 33	HPV 33
49	HPV 33	HPV 33

50	HPV 51	HPV 18
51	HPV 16	HPV 16
52	HPV 33	HPV 33
53	HPV 58	HPV58
54	HPV 16, 18	HPV 16, 18
57	HPV 58	HPV 58
58	HPV 33	HPV 33
59	HPV 18	HPV 18
60	HPV 18	HPV 18
62	HPV 39	-
63	HPV 35	HPV 35
64	-	-
65	HPV 58	HPV 58
66	-	-
68	No typing	HPV 52, 58
69	No typing	-
70	HPV 16	HPV 16
71	HPV 16	HPV 16
72	-	-
73	HPV 16	HPV 16
75	-	HPV 16
76	HPV 16	HPV 16
77	HPV 18	HPV 16
78	HPV 16	HPV 16
79	HPV 33, 35	HPV 33
80	HPV 33	HPV 33
81	HPV 16	HPV 16
82	HPV 16	HPV 16
83	-	HPV 33
84	HPV 16	HPV 16
85	HPV 16	HPV 16
86	HPV 16	HPV 16
87	HPV 16	HPV 16
88	HPV 16	HPV 16
89	HPV 58	HPV 58
90	HPV 16	HPV 16
91	HPV 16	HPV 16
92	HPV 16	HPV 16
93	HPV 16	HPV 16
94	HPV 16	HPV 16
95	HPV 16	HPV 16
96	-	HPV 33
97	HPV 16	HPV 16
98	HPV 16	HPV 16
99	HPV 16	HPV 16
100	HPV 16	HPV 16

101	HPV 16	HPV 16
102	HPV 16	HPV 16
103	HPV 16	HPV 16
104	HPV 16	HPV 16
105	HPV 16	HPV 16
106	HPV 16	HPV 16
107	HPV 16	HPV 16
108	HPV 16	HPV 16
109	HPV 16	HPV 16
110	HPV 16	HPV 16
111	HPV 18	HPV 18
112	HPV 16	HPV 16
113	HPV 16	HPV 16
114	HPV 16	HPV 16
115	HPV 31, 35	HPV 31
116	HPV 16	HPV 16
117	HPV 16	HPV 16
118	HPV 16	HPV 16
119	HPV 16	HPV 16
120	HPV 16	HPV 16
121	HPV 16	HPV 16
123	HPV 16	HPV 16
124	HPV 51	HPV 31

In Table 1, "no typing" indicates presence of HPV DNA after PCR amplification with HPV type undetermined by the specific method, and "-" indicates absence of HPV DNA after PCR amplification. As shown in Table 1, the results obtained by two methods under comparison were in a good accordance demonstrating the reliability of DNA chip analysis. Considering the simplicity and rapidity of procedure, together with convenient detection of diverse genotypes and multiple infection, DNA chip analysis is thought to be by far more advantageous than PCR-RFLP followed by type-specific PCR and other related methods. The accuracy of diagnosis by the DNA chip of the invention was calculated to be 96.5% and the reproducibility was 95% based on the above 124 cases, which are considered to be the subject of improvement upon completion of larger size case studies in progress. The FDA(Food and Drug Administration) approved Hybrid Capture kit increasingly

employed recently for fast diagnosis of HPV infection was reported to have 98% accuracy in detecting and distinguishing high- or low-risk HPV infection. DNA chip analysis has a competitive efficiency and an additional advantage of genotyping when compared with the Hybrid Capture assay. The above information indicates that diagnosis of HPV infection using the genotyping kit of the invention is superior in many aspects to the conventional methods employed for the same purpose.

As clearly illustrated and demonstrated as aboves, the present invention provides a genotyping kit for identifying genotypes of HPV from clinical samples of infected patients and a method for diagnosis of HPV infection by genotyping the infecting virus using the said genotyping kit. The HPV genotyping kit of the invention comprises: a DNA chip with probes that have nucleotide sequences complementary to DNA of HPV; primers for amplifying DNA obtained from clinical samples by PCR; and, means for labeling amplified DNA hybridized with the probes of the said DNA chip. The method for diagnosis of HPV infection using the said HPV genotyping kit comprises the steps of: amplifying DNA obtained from clinical samples by PCR with primers of the kit; applying the amplified DNA to DNA chip to hybridize the amplified DNA with the probes of the DNA chip; and, detecting DNA bound on the surface of the DNA chip after labeling DNA hybridized with the probes of the DNA chip with means of labeling of the HPV genotyping kit. HPV genotyping kit of the invention is an implement that can detect HPV infection in a simple and accurate manner, as well as identify the types of infecting HPV, therefore, it may contribute to early diagnosis, prevention and treatment of cervical cancer.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing descriptions.

Such modifications are also intended to fall within the scope of the appended claims.

What is claimed is:

1. A Human Papillomavirus(HPV) genotyping kit which comprises:

(i) a DNA chip with probes that have nucleotide sequences complementary to DNA of HPV;

(ii) primers for amplifying DNA obtained from clinical samples by PCR; and,

(iii) means for labeling amplified DNA hybridized with the probes of the said DNA chip.

2. The HPV genotyping kit of claim 1 wherein the DNA chip further comprises position markers to locate probes.

3. The HPV genotyping kit of claim 1 wherein the primers are selected from the group consisting of GP5+ having Sequence ID No. 22, GP6+ having Sequence ID No. 23, GP5d+ having Sequence ID No. 24 and GP6d+ having Sequence ID No. 25.

4. The HPV genotyping kit of claim 1 wherein the means for labeling is a biotin-binding material.

5. The HPV genotyping kit of claim 4 wherein the biotin-binding material is streptavidin-R-phycoerythrin.

6. A process for preparing a DNA chip which comprises the steps of:

(i) preparing 5' terminal amine-linked DNA probes which have nucleotide sequences complementary to DNA of HPV;

(ii) affixing the DNA probes thus prepared to an aldehyde-derivatized surface of solid support; and

(iii) reducing excessive aldehydes not reacted with amine.

7. The process for preparing DNA chip of claim 6

wherein the concentration of probes which react with aldehyde-derivatized solid surface ranges from 100 to 300pmol/μ l.

8. The process for preparing DNA chip of claim 6 wherein affixing DNA probes to aldehyde-derivatized solid surface is performed via Schiff's base reaction between amine and aldehyde groups under an environment of 30 to 40 C and 70 to 100% humidity.

9. The process for preparing DNA chip of claim 6 wherein the reduction of aldehyde is performed by the aid of a reducing agent, NaBH .

10. A method for diagnosis of HPV infection using a HPV genotyping kit which comprises the steps of:

(i) amplifying DNA obtained from clinical samples by PCR with primers of HPV genotyping kit of claim 1 to give biotin-containing amplified DNA;

(ii) applying the amplified DNA thus obtained to DNA chip of the HPV genotyping kit to hybridize the amplified DNAs with DNA probes of the DNA chip; and,

(iii) detecting DNA bound on the surface of the DNA chip after labeling amplified DNA hybridized with the probes with means for labeling of the HPV genotyping kit.

11. The method for diagnosis of HPV infection using a HPV genotyping kit of claim 10 wherein the amplification of DNA obtained from clinical samples is performed by PCR using biotin-16-dUTP.

ABSTRACT

The present invention relates to a genotyping kit for diagnosing patients infected with human papillomavirus(HPV), and a method for diagnosis of HPV infection by genotyping specimen DNA isolated from the patients using the said kit. The genotyping kit of the invention comprises a DNA chip with probes that have nucleotide sequences complementary to DNA of HPV, primers for amplifying the DNA of a sample by PCR, and means for labeling hybridized sample DNA with the DNA chip. The method for diagnosis of HPV infection comprises the steps of amplifying DNA obtained from a sample using the primers of the kit, applying the amplified DNA to the DNA chip and hybridizing the amplified DNA and the probes of the DNA chip, and detecting DNA bound on the surface of the DNA chip by labeling hybridized DNA. In accordance with the invention, the genotyping kit may be practically applied to the early diagnosis, prevention and treatment of cervical cancer, since the kit can easily diagnose HPV infection, and can exactly determine the genotype of the HPV.

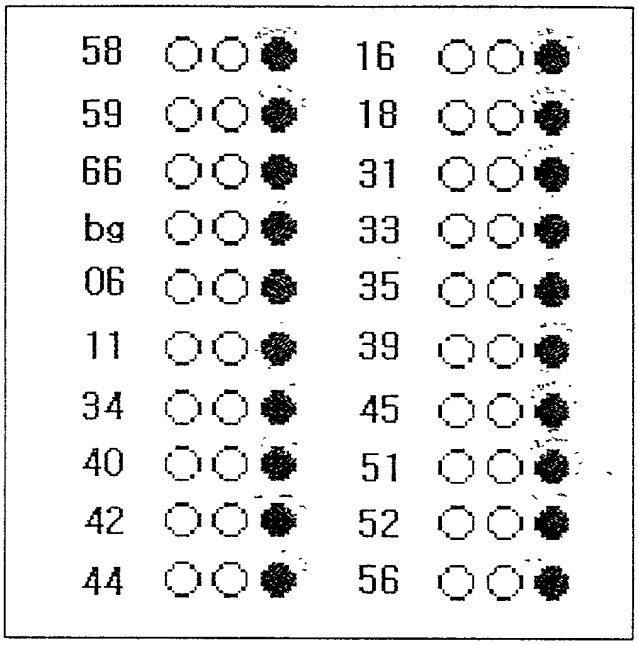

Fig. 1

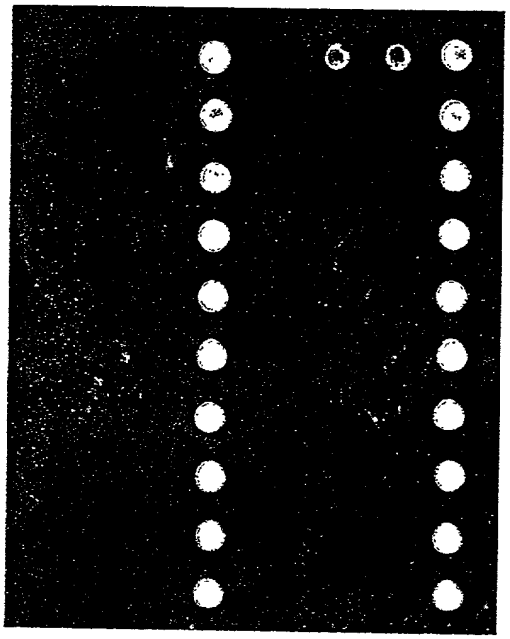

Fig. 2a

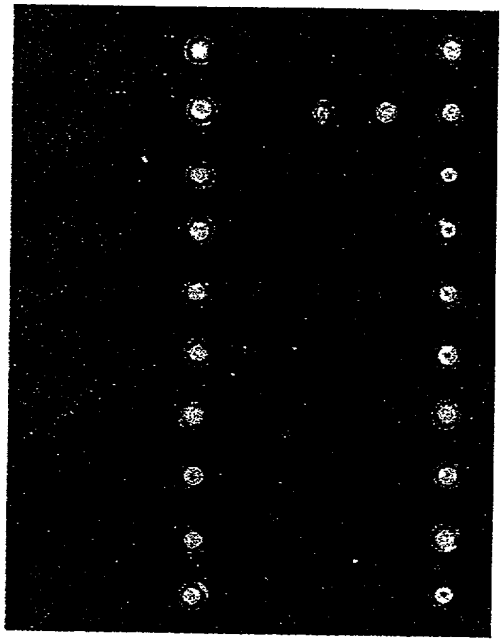

Fig. 2b

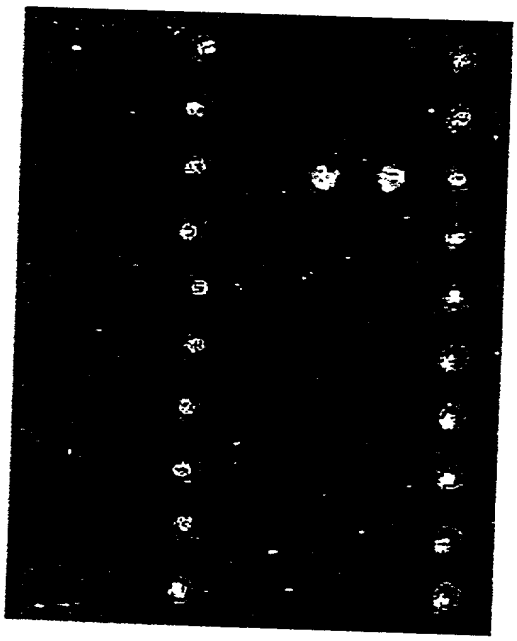

Fig. 2c

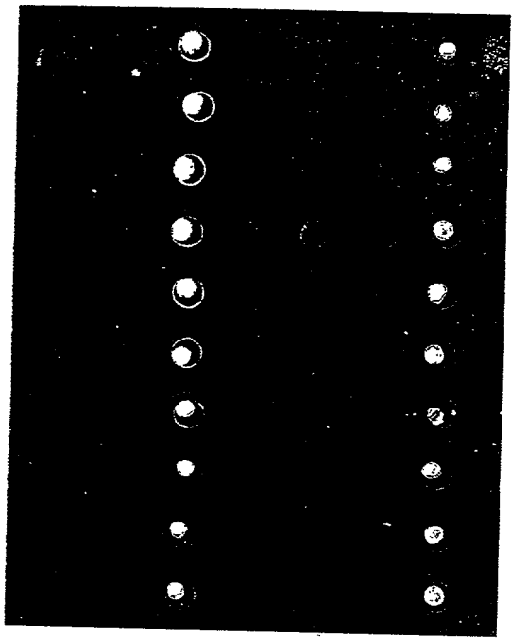

Fig. 2d

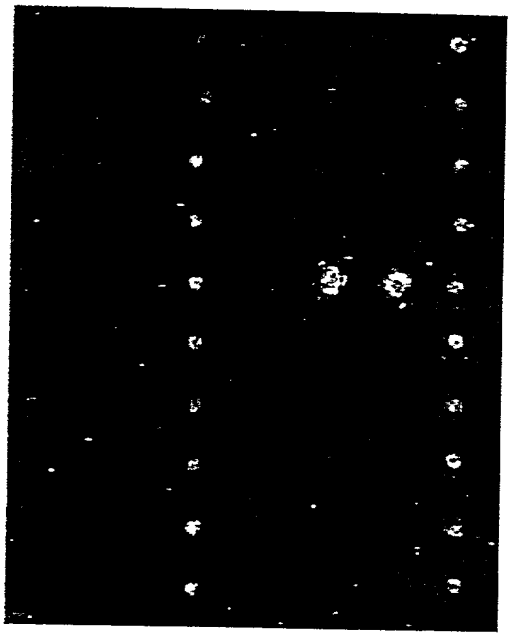

Fig. 2e

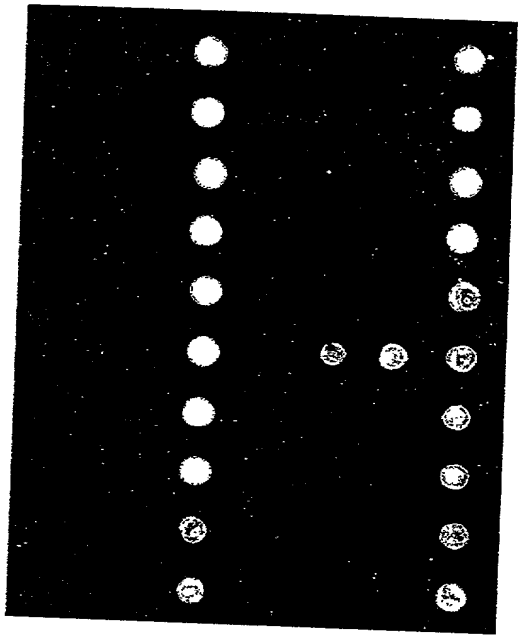

Fig. 2f

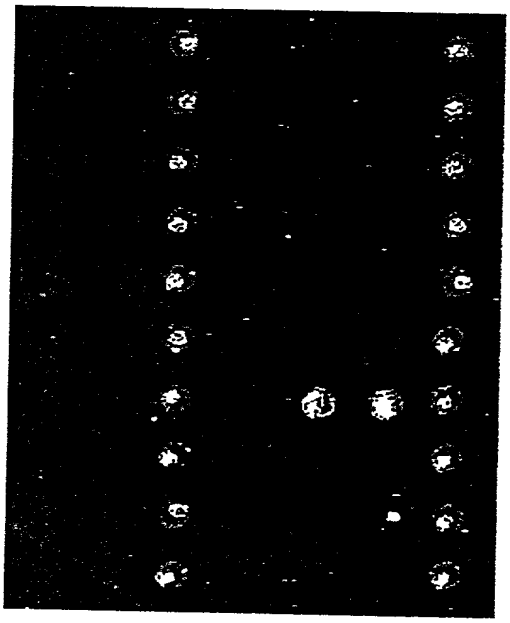

Fig. 2g

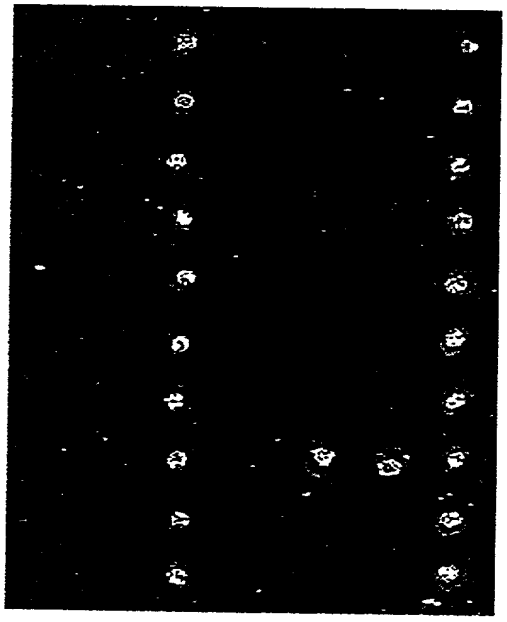

Fig. 2h

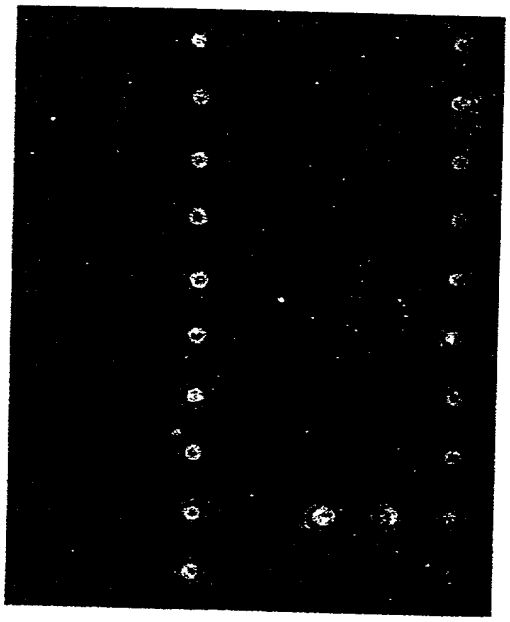

Fig. 2i

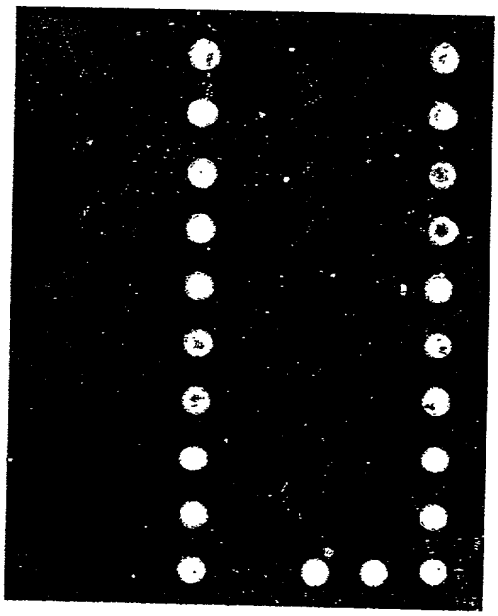

Fig. 2j

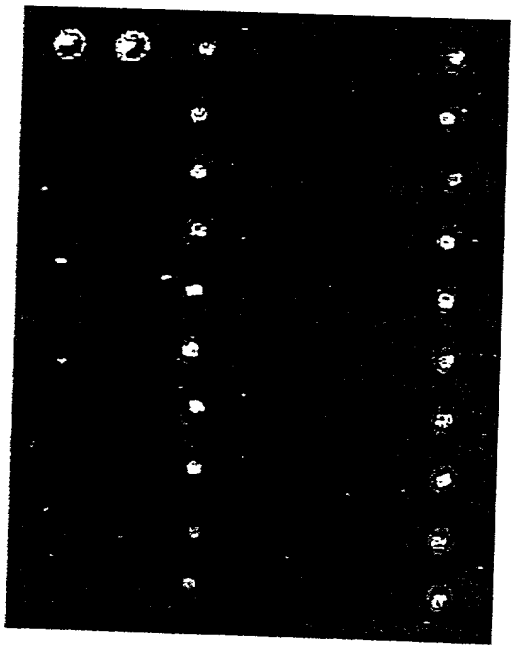

Fig. 2k

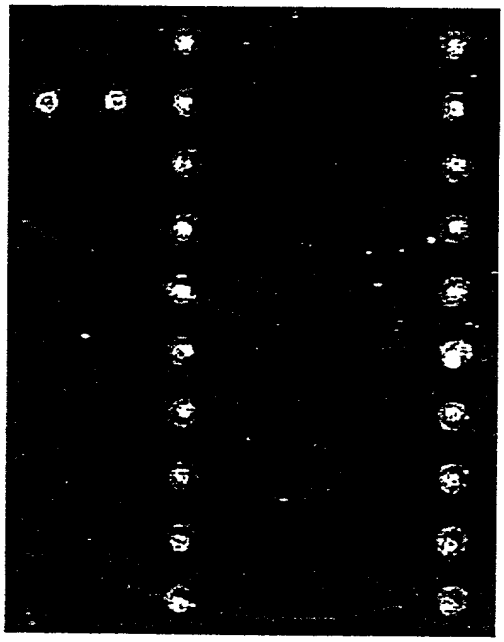

Fig. 2l

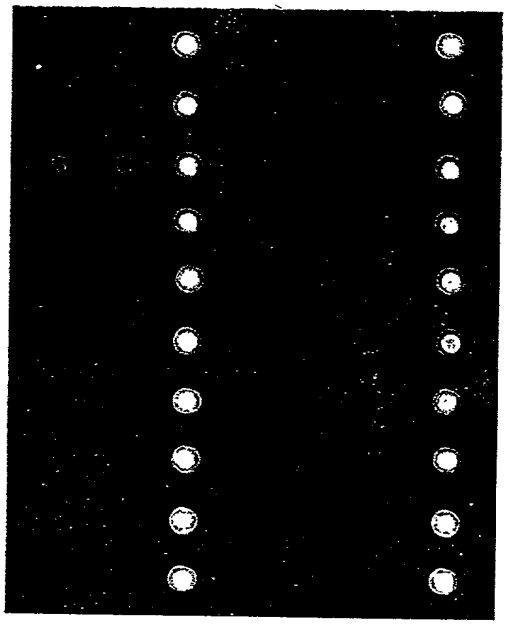

Fig. 2m

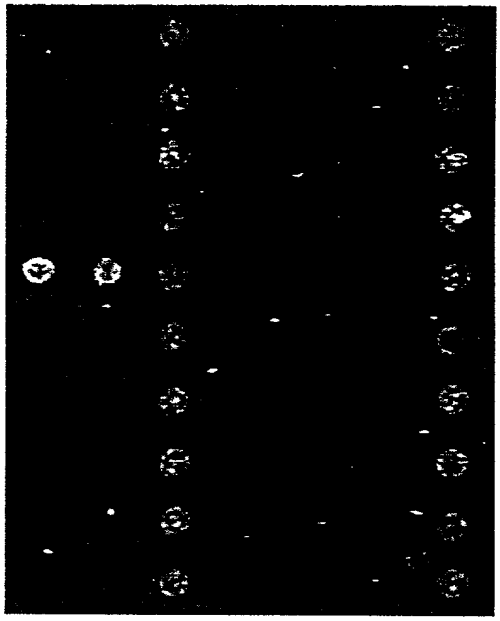

Fig. 3a

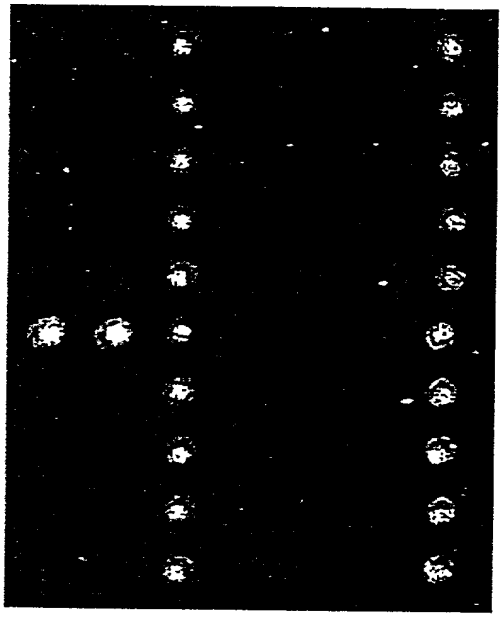

Fig. 3b

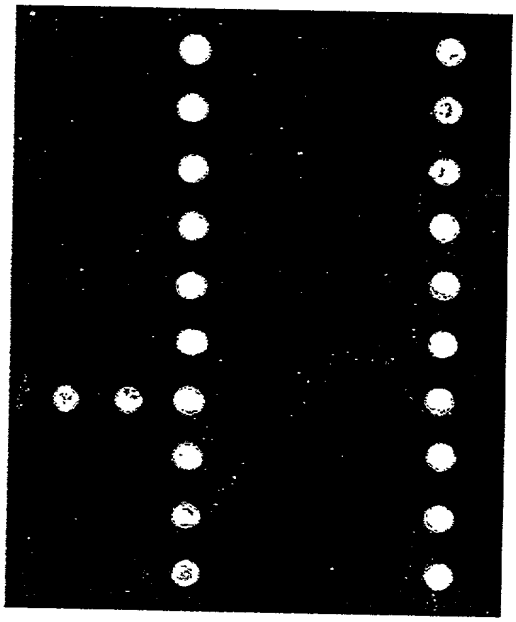

Fig. 3c

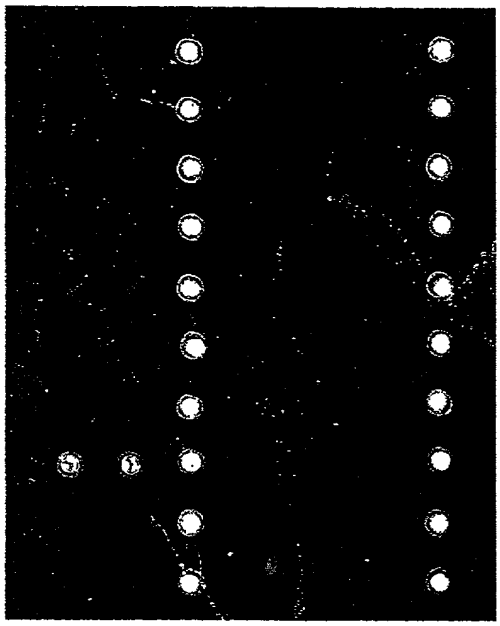

Fig. 3d

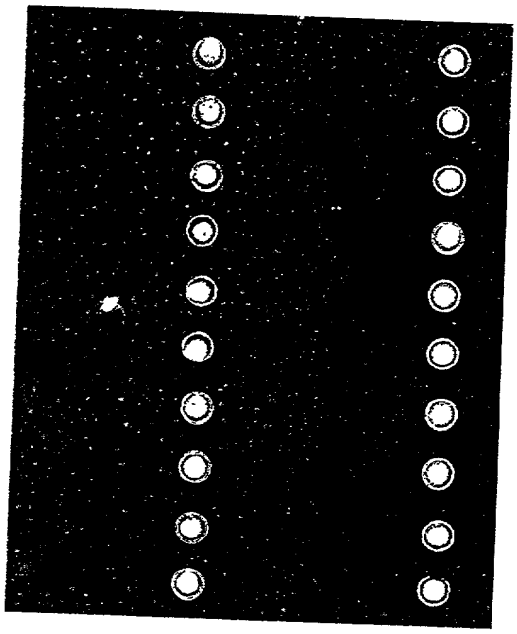

Fig. 3e

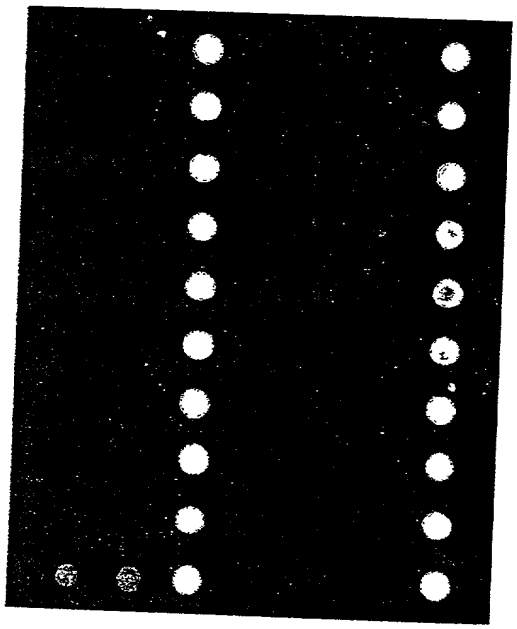

Fig. 3f

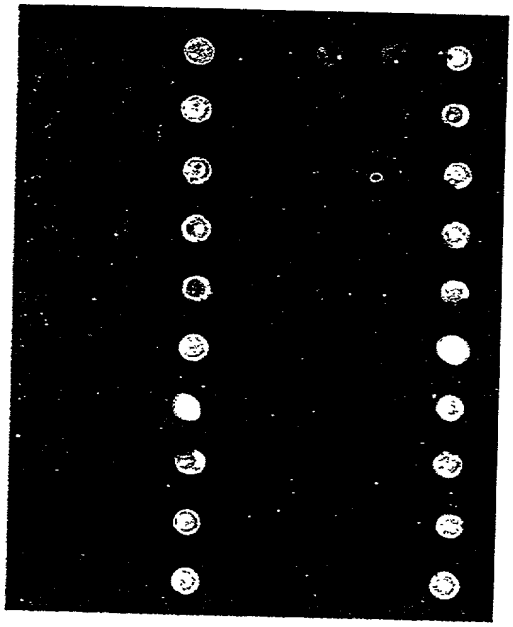

Fig. 4a

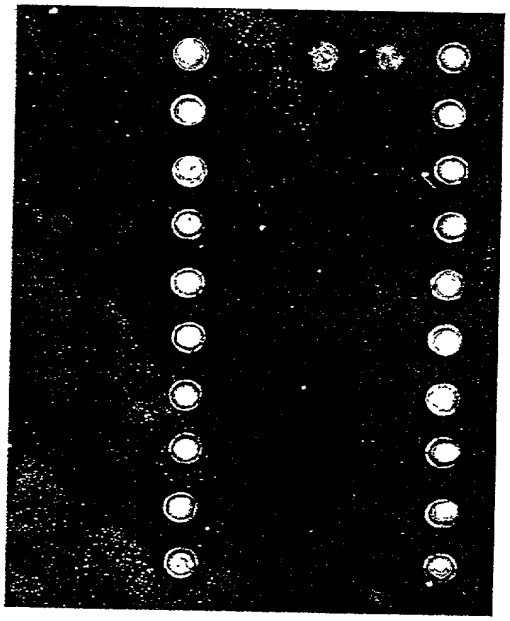

Fig. 4b

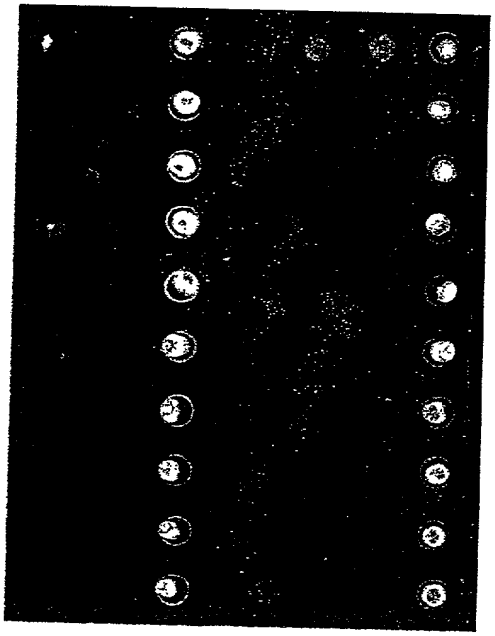

Fig. 4c

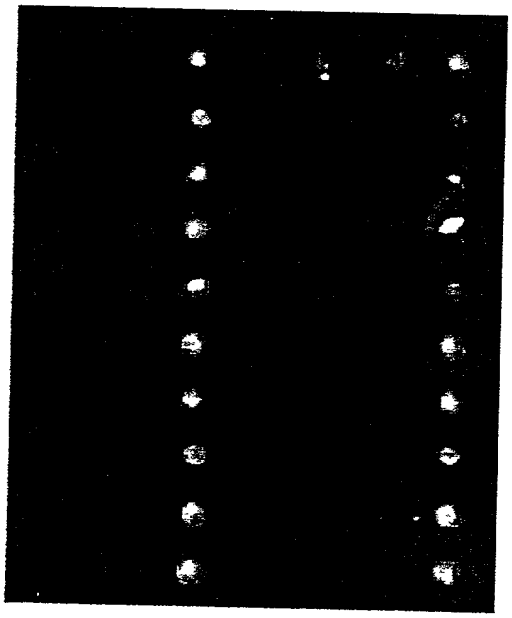

Fig. 4d

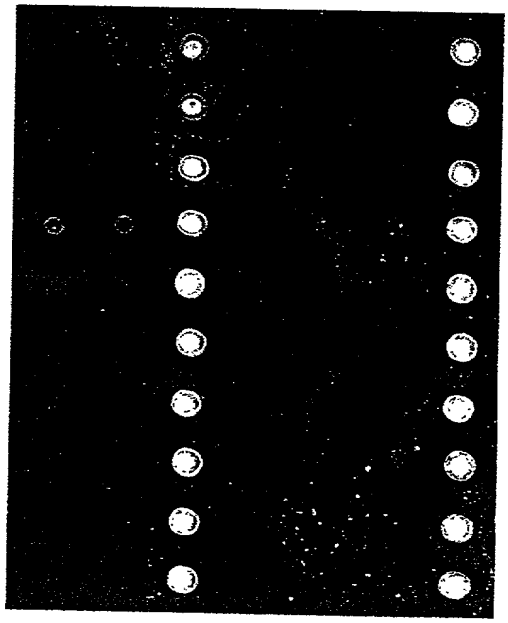

Fig. 4e

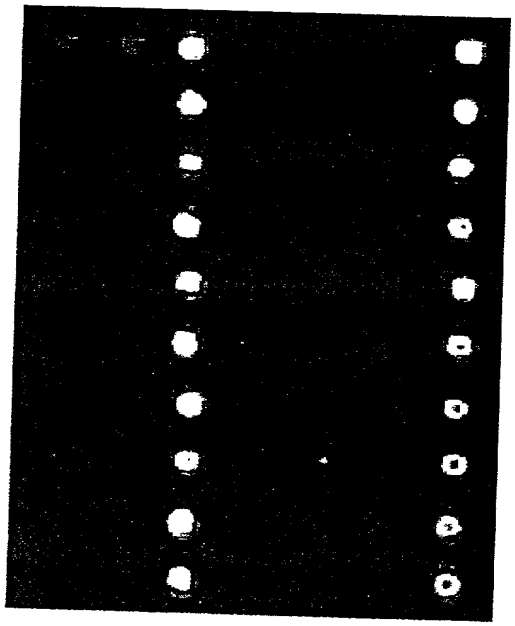

Fig. 4f

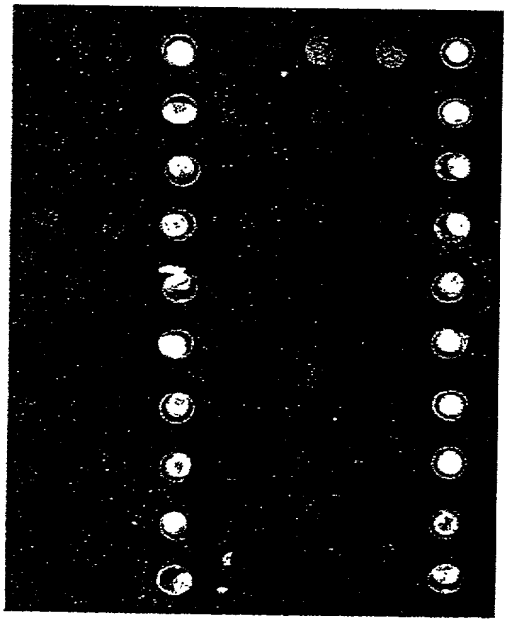

Fig. 4g

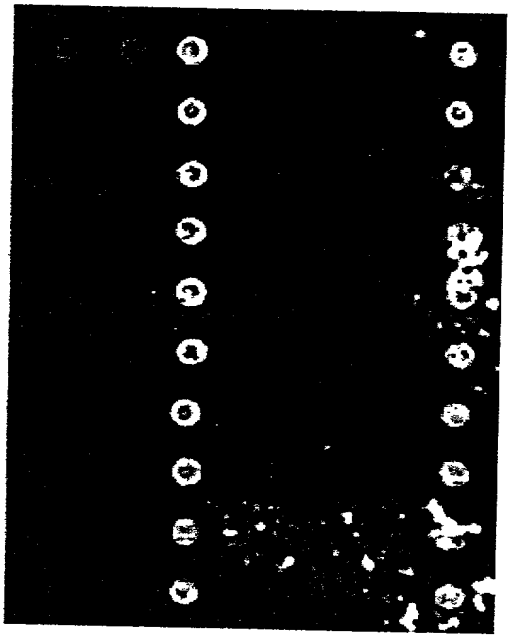

Fig. 4h

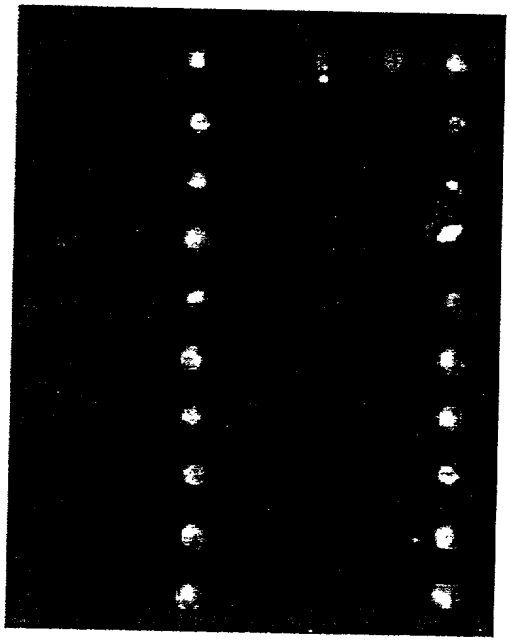

Fig. 4i

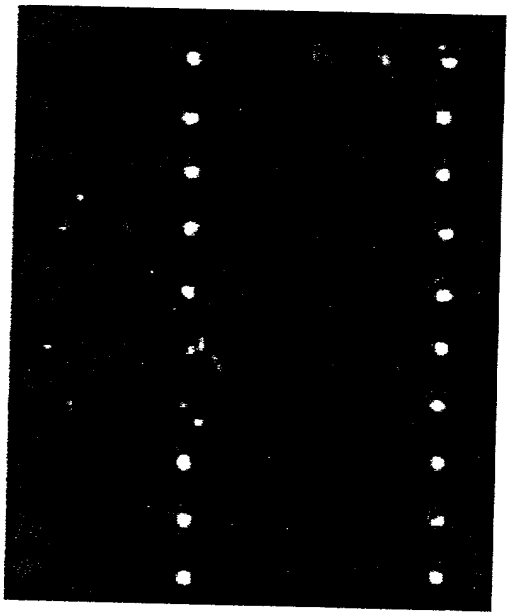

Fig. 4j

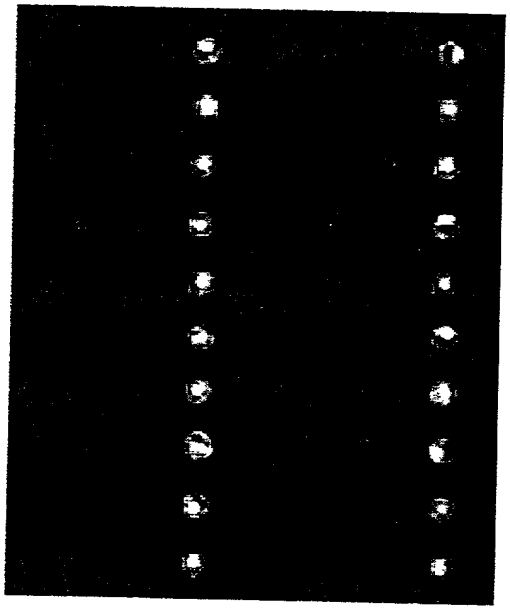

Fig. 4k

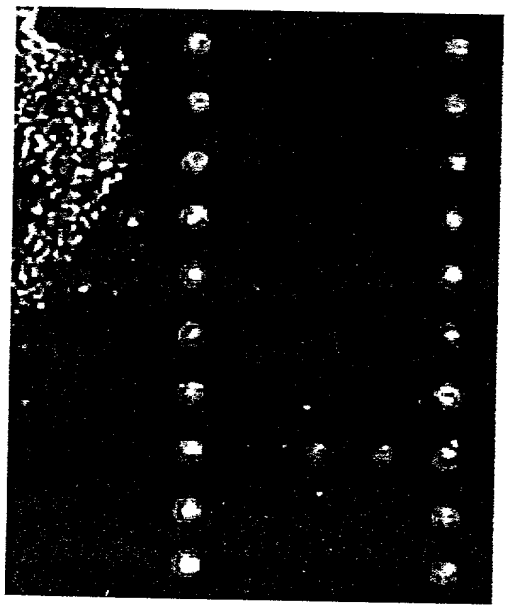

Fig. 4l

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

As below named inventors, we hereby declare that:

Our residences, post office addresses and citizenships are as stated below next to our names. We believe we are the original, first and joint inventors of the subject matter which is claimed for and which a patent is sought on the invention entitled:

"GENOTYPING KIT FOR DIAGNOSIS OF HUMAN PAPILLOMAVIRUS INFECTION

the specification of which is attached hereto.

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by us on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119

COUNTRY (if PCT Indicate PCT)	APPLICATION NO.	DATE OF FILING (day/month/year)	PRIORITY CLAIMED
Korea	2000-13161	15/03/00	Yes
PCT	PCT/00/01213	26/10/00	NO

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, Morris Relson #15,108, Gordon D. Coplein #19,165, William F. Dudine, Jr. #20,569, Michael J. Sweedler #19,937, S. Peter Ludwig #25,351, Paul Fields #20,298, Joseph B. Lerch #26,936, Melvin C. Garner #26,272, Ethan Horwitz #27,646, Beverly B. Goodwin #28,417, Adda C. Gogoris #29,714, Martin E. Goldstein #20,869, Bert J. Lewen #19,407, Henry Sternberg #22,408, Peter C. Schechter #31,662, Robert Schaffer #31,194, David R. Francescani #25,159, Robert C. Sullivan, Jr., #30,499

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We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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KICX

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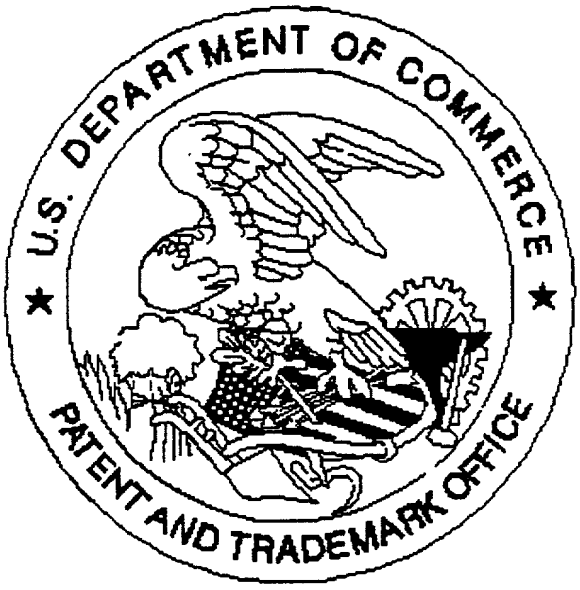

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☑ ***Scanned copy is best available.*** Drawings.